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Kinetic Analysis of Lactose and Proton Coupling in Glu^{379} Mutants of the Lactose Transport Protein of *Streptococcus thermophilus**

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The role of Glu^{379} in the lactose-H$^+$ symport protein (LacS) of *Streptococcus thermophilus* was studied by analyzing the kinetic mechanism of transport of wild-type and Ala^{379}, Asp^{379}, and Gln^{379} mutant proteins. Glu^{379} forms part of the sequence motif Lys-*X*-*X*-His-*X*-*X*-Glu that is present in a number of sugar transport proteins, including LacY of *Escherichia coli*. The E379A and E379Q mutants were defective in the uptake of lactose against a concentration gradient and lactose-dependent proton uptake, but catalyzed facilitated influx of lactose down a concentration gradient and equilibrium exchange with rates similar to that of the wild-type enzyme. The E379D mutant was partially defective in the coupled transport of lactose and protons. These results suggest that an acidic residue at position 379 is required for the coupled uptake of lactose and protons and are consistent with a mechanism in which lactose transport in the E379A and E379Q mutants occurs by uniport rather than proton symport. Lactose efflux down a concentration gradient in wild-type LacS and LacS-E379D increased with pH with apparent pK (pK_a) values of ≥8.5 and 8.0, respectively, whereas efflux in the E379Q mutant increased sigmoidally with a pK_a of about 6.0. Imposition of an artificial membrane potential (inside negative) in membrane vesicles bearing wild-type LacS or LacS-E379Q not only inhibited the lactose efflux mediated by wild-type but also that of the mutant enzyme. To associate the role of Glu^{379} with specific step(s) in the translocation cycle of LacS, the properties of wild-type LacS and the Glu^{379} mutants have been evaluated by numerical analysis of simple kinetic schemes for translocation catalysis by solute H$^+$ symport proteins. The properties of the wild-type enzyme are consistent with a mechanism in which the order of ligand binding on the inside is substrate first and proton last, whereas the order is random (or proton first, substrate last) at the outer surface of the membrane. The wild-type enzyme is asymmetric with regard to proton binding; the pK for proton binding on the outside is at least 4 units higher than the pK on the inside. The properties of the Glu^{379} mutants correspond with a lowering of the pK on the outside (pK_{OUT} ~ pK_{IN}), and the induction of a leak pathway in which the binary enzyme-substrate complex becomes mobile.

The lactose transport protein (LacS) of *Streptococcus thermophilus* catalyzes the uptake of galactosides in symport with a proton (Foucaud and Poolman, 1992). *In vivo* the dominant transport reaction corresponds with an exchange of lactose for intracellularly formed galactose without net movement of protons (Poolman, 1990; Foucaud and Poolman, 1992). The structure of the LacS protein differs from other secondary transport proteins by the presence at the carboxyl terminus of a hydrophilic domain that is homologous to IIA of various phosphoenolpyruvate:sugar phosphotransferase systems (Poolman *et al.*, 1989). Although the IIA domain of LacS is not essential for translocation catalysis, phosphorylation of a histidine residue in the IIA domain by phosphoenolpyruvate and the general energy coupling proteins of the phosphoenolpyruvate:sugar phosphotransferase systems reduces the transport activity (Poolman *et al.*, 1992, 1995).

The amino-terminal carrier domain of LacS corresponds with a polytopic membrane protein that traverses the membrane most likely 12 times. The primary sequence of the hydrophobic carrier domain of LacS classifies this protein as a member of a family which also includes MelB of *Escherichia coli, Klebsiella pneumoniae*, and *Salmonella typhimurium*, the glucuronide transporter (GusB) of *E. coli* (Liang, 1992), and the xylose transporter (XylP) of *Lactobacillus pentosus* (for a review, see Poolman and Konings, 1993). The lactose transport protein (LacY) of *E. coli* is not homologous to members of this family of transporters, however, a stretch of about 20 amino acids around His^{322} in LacY can be identified in LacS (Fig. 1). Conserved residues include a lysine (position 319 in LacY, 373 in LacS; *arrow 1*), a histidine (position 322 in LacY, 376 in LacS; *arrow 2*), and a glutamic acid (position 325 in LacY, 379 in LacS; *arrow 3*). Residues His^{322}, Glu^{325} (which should be on the same side of putative α-helix X), and Arg^{302} (putative helix IX) of LacY have been proposed to participate in proton translocation via a charge relay mechanism (Püttner *et al.*, 1986, 1989; Carrasco *et al.*, 1986; Menick *et al.*, 1987; Lee *et al.*, 1989), which is based on analogies with proton transfer via Asp, His, and Ser in serine type proteases (Kraut, 1977). In this view, Glu^{325} and His^{322} are ion-paired and interact with Arg^{302}; Glu^{325} and Arg^{302} would polarize the imidazole group of His^{322} which enhances its capacity to act as a proton shuttle. His^{322} is poised to accept a proton from Glu^{325} and subsequent transfer of this proton to Arg^{302}, another residue or the medium would lead to net proton translocation. The role of His^{322} in proton translocation can be questioned since the requirement for an ionizable histidine residue at position 322 in LacY is not always needed for galactoside accumulation and galactoside-dependent proton transport (King and Wilson, 1989a, 1989b, 1990; Franco and Brooker, 1991). Similarly, some substitutions of Arg^{302}, *i.e.* R302S and R302H, result in mutant proteins that do accumulate galactosides albeit to lower levels and do exhibit sugar-dependent proton transport (Matzke *et al.*, 1992). The degree of uncoupling of sugar transport from H$^+$ transport not

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										⇓1				⇓2			⇓3													
$LacY_{EC}$	311-339	S	A	L	E	V	V	I	L	**K**	T	L	**H**	M	F	E	V	P	F	L	L	V	G	C	F	K	Y	I	T	S
$LacY_{KP}$	316-344	S	A	V	E	V	V	I	L	**K**	M	L	**H**	M	F	E	I	P	F	L	L	V	G	T	F	K	Y	I	S	S
$RafB_{EC}$	314-342	T	M	T	E	V	V	I	L	**K**	M	L	**H**	A	L	E	V	P	F	L	L	V	G	A	F	K	Y	I	T	G
$CscB_{EC}$	311-339	N	P	W	I	I	S	L	V	**K**	L	L	**H**	A	I	E	V	P	L	C	V	I	S	V	F	K	Y	S	V	A
						:			:	#			#			#				:		:		:						
$LacS_{ST}$	365-393	**D**	S	**V**	**E**	**Y**	**G**	Q	W	**K**	T	G	**H**	**R**	D	**E**	S	L	T	L	**S**	V	R	P	L	I	D	**K**	L	**G**
$LacS_{LB}$	358-386	**D**	A	**V**	**E**	**Y**	**G**	Q	L	**K**	T	G	**H**	**R**	D	**E**	A	L	T	L	**S**	V	R	P	L	I	D	**K**	L	**G**
$RafP_{PP}$	386-414	**D**	S	**V**	**E**	**Y**	**G**	Q	L	**K**	L	G	**H**	**R**	D	**E**	S	L	A	L	**S**	V	R	P	L	I	D	**K**	F	**G**
		#		#	:	#	#	:						#		#		:			#	:			:			#		#
$MelB_{EC}$	347-375	**D**	I	**V**	**D**	**Y**	**G**	E	Y	**K**	L	H	V	**R**	C	**E**	S	I	A	Y	**S**	V	Q	T	M	V	V	**K**	G	**G**
$MelB_{SY}$	351-379	**D**	T	**V**	**D**	**Y**	**G**	E	F	**K**	L	N	I	**R**	C	**E**	S	I	A	Y	**S**	V	Q	T	M	V	V	**K**	G	**G**
$MelB_{KP}$	351-379	**D**	T	**V**	**D**	**Y**	**G**	E	Y	T	M	N	I	**R**	C	**E**	S	I	A	Y	**S**	V	Q	T	L	V	V	**K**	A	**G**
$GusB_{EC}$	345-373	**D**	T	**V**	**E**	**Y**	**G**	E	Y	L	T	G	V	**R**	I	**E**	G	L	T	Y	**S**	L	F	S	F	T	R	**K**	C	**G**
$XylP_{LP}$	367-395	**D**	S	**V**	**D**	**Y**	**G**	E	W	**K**	N	G	V	**R**	A	**E**	G	I	V	T	**S**	F	S	S	F	S	A	**K**	F	**G**

FIG. 1. **Conserved residues in interhelix loop X-XI of LacS and other sugar transport proteins.** *LacY*, lactose transport protein; *RafB*, raffinose transport protein; *CscB*, sucrose transport protein; *LacS*, lactose transport protein; *MelB*, melibiose transport protein; *GusB*, glucuronide transport protein; *XylP*, xylose transport protein. The subscripts *EC*, *KP*, *ST*, *LB*, *SY*, and *LP* refer to *E. coli*, *K. pneumoniae*, *S. thermophilus*, *Lactobacillus bulgaricus*, *S. typhimurium* and *Lactobacillus pentosus*, respectively (for details, see Poolman and Konings, 1993). Lys^{373}, His^{376}, and Glu^{379} of LacS are indicated by the *arrows 1–3*, respectively.

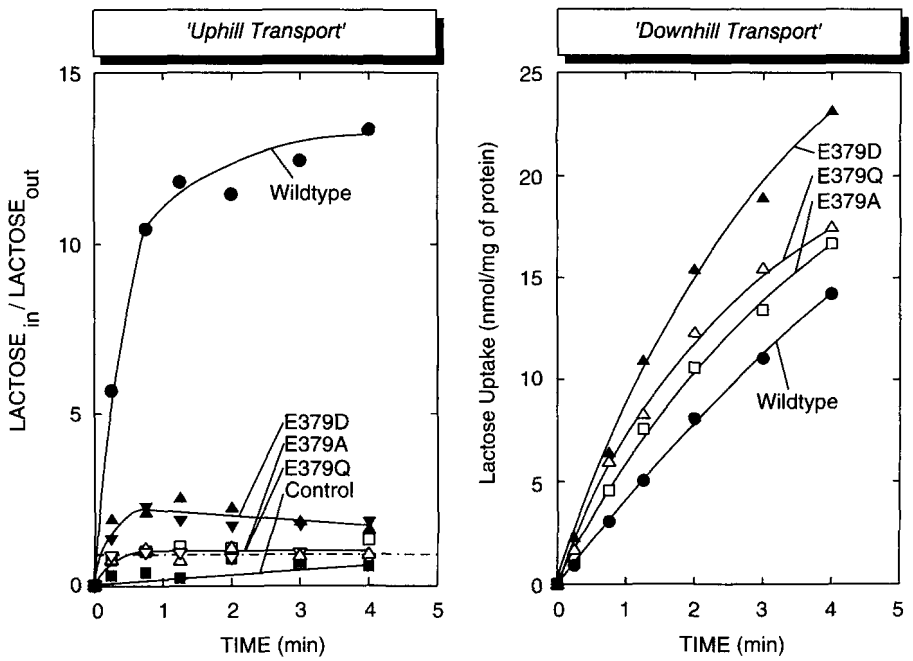

FIG. 2. **Uphill and downhill transport of lactose.** For uphill transport, *E. coli* DW2/pSKE8 wild-type and various LacS mutants were grown in Luria broth supplemented with carbenicillin (50 μg/ml). Cells were washed and resuspended in KPM, pH 6.6, to a final protein concentration of 60–70 mg/ml. Transport of lactose (19 μM, final concentration) was assayed in the presence of the electron donor D-Li-lactate and at a final protein concentration of 0.6–0.7 mg/ml. For downhill transport, *E. coli* HB101/pSKE8 wild-type and various mutants were grown in Luria broth supplemented with carbenicillin (50 μg/ml) and 0.1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were treated as described above, and transport of lactose (47 μM, final concentration) was assayed in the absence of exogenous electron donor. The experiments were performed at 30 °C.

only varies with the substitutions made for Arg^{302} and His^{322}, but also with the sugar used in the transport assay (King and Wilson, 1989a, 1989b) and other parameters such as the pH of the assay medium (Franco and Brooker, 1991). Altogether, one could conclude that Arg^{302} and His^{322} play an auxiliary role in proton translocation, *e.g.* via modulating the p*K* of an essential nearby residue. From the analysis of LacY Glu^{325} mutants, it is clear that an acidic residue at position 325 is required for proton-linked galactoside transport (Carrasco *et al.*, 1989; Franco and Brooker, 1994).

In this study, we focus on the role in translocation of Glu^{379} in the lactose transport protein of *S. thermophilus*. The data indicate that Glu^{379} is required for the coupled uptake of lactose and protons possibly by modulating the p*K* of an essential protonatable residue. The properties of the *S. thermophilus* LacS(Glu^{379}) mutant proteins are highly diagnostic of an enzyme with a substrate leak pathway that allows uncoupled transport at rates similar to or better than the coupled transport by the wild-type enzyme.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Growth Conditions, and Preparation of Cell Suspensions

The bacterial strains used are *E. coli* DW1 (*Δlac(ZY), Δmel(AB), strA*); DW2 (*lacI*$^+$, *Δlac(ZY), melA*$^+$, *ΔmelB, strA*); JM101 (*supE, thi Δ(lac-proAB), [F', traD36, proAB, lacI*^{q}*ZΔM15]*); CJ236 (*dut1, ung1, thi1, relA1*/pCJ105 (Cm^R)); HB101 (*hsdS20(r*$_B^-$*m*$_B^-$*B), recA13, ara14, proA2, lacY1, galK2, rps* (Sm^R), *xyl5, mtl1, supE44*, λ^-, F^-). *E. coli* cells were grown aerobically at 37 °C in Luria broth, M9 mineral medium supplemented with 4 g/liter casamino acids and 50 mM glucose or lactose or M9 mineral medium supplemented with 50 mM carbohydrate and essential nutrients as indicated by the auxotrophic markers (Sambrook *et al.*, 1989). When appropriate, the medium was supplemented with carbenicillin (50 μg/ml), ampicillin (100 μg/ml), chloramphenicol (10 μg/ml), and/or streptomycin (25 μg/ml). Overnight cultures or exponentially growing cells (OD_{660} = 0.4–0.8; Erlenmeyer flasks) were harvested by centrifugation, washed twice, and resuspended to a final protein concentration of about 40 mg/ml in 100 mM potassium phosphate 2 mM magnesium sulfate (KPM buffer) or 30 mM K-citrate, 30 mM K-piperazine-*N,N'*-bis(2-ethanesulfonic acid), 30 mM K-2(*N*-cyclohexylamine)ethanesulfonic acid, 2 mM $MgSO_4$ (CPC buffer) (pH 6.5, unless indicated otherwise). Concentrated cell suspensions

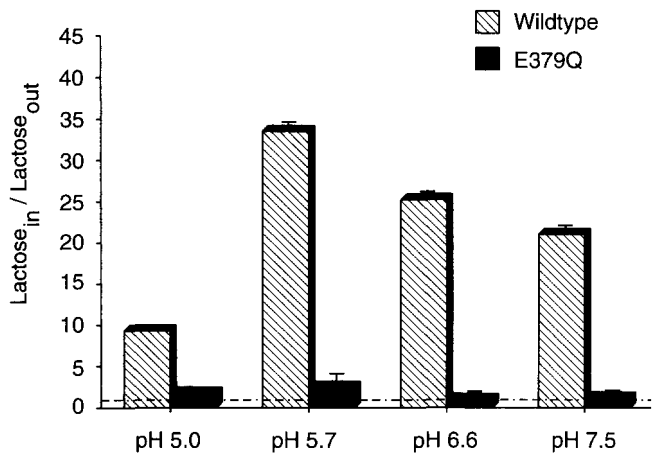

FIG. 3. **Steady state accumulation levels of lactose in *E. coli* DW2/pSKE8 LacS (wild-type), and LacS-E379Q.** For experimental procedures see legend to Fig. 2 (uphill transport), except that cells were washed and resuspended in KPM of the indicated pH and transport was assayed at a final lactose concentration of 3.6 μM. Uptake of lactose was monitored for 30 min and samples were taken every 1–2 min.

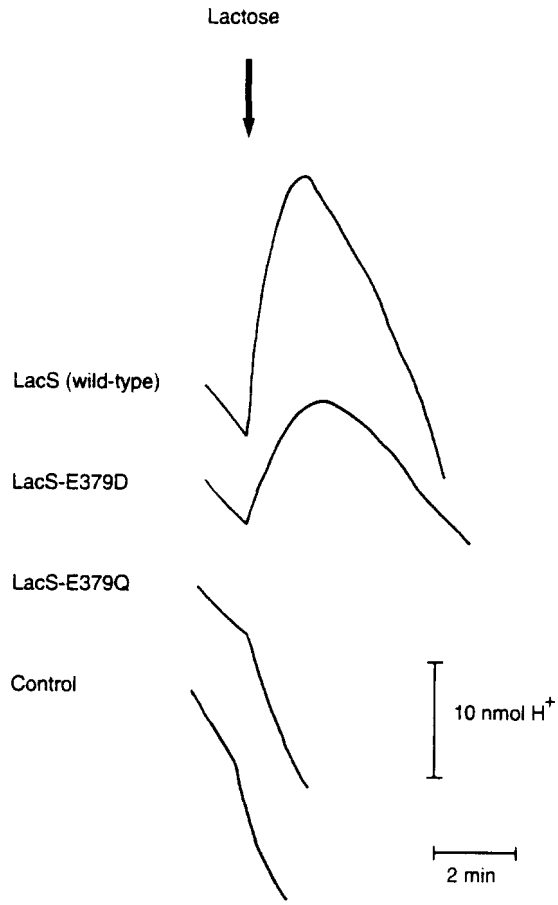

FIG. 4. **Lactose-induced proton uptake by LacS (wild-type), LacS-379D, and LacS-E379Q.** *E. coli* DW2/pSKE8 was grown in M9 mineral medium supplemented with 1 g/liter casamino acids, 0.001% (w/v) thiamine, 20 mM glycerol, and 50 μg/ml carbenicillin. Lactose (30 μl of a 500 mM solution) was added at the time indicated by the *arrow*. The pH of the suspension was monitored with an electrode; small aliquots of 10 mM KOH were used to calibrate the pH recordings.

were stored on ice until use.

Isolation of Membranes—For the isolation of right-site-out membrane vesicles cells were grown in 10–15-liter fermentors with vigorous aeration using M9 mineral medium supplemented with 4 g/liter casamino acids and 50 mM glucose. The cells grew exponentially up to optical densities at 660 nm of 2.5. Cells were harvested at OD_{660} of 2.0–2.5, and right-site-out membrane vesicles were prepared according to Kaback (1971). Inside-out membrane vesicles were prepared by a 2-fold passage of the cells through a French pressure cell (20,000 pounds/square inch) as described (Poolman *et al.*, 1983).

Transport Assays—Transport experiments were performed at 30 °C unless specified otherwise.

(i) Active Transport—Cells (0.6–1.2 mg/ml) in KPM buffer containing 10 mM D-Li-lactate as the electron donor were preenergized for 2 min in the presence of oxygen. At time 0, radiolabeled substrate was added, and at appropriate time intervals the uptake reaction was stopped by addition of 2 ml of ice-cold 100 mM LiCl. Cells were filtered (0.45-μm cellulose nitrate filters (Millipore Corp.)) and washed with 2 ml of ice-cold LiCl. For the estimation of intracellular concentrations, a specific internal cell volume of 3 μl/mg of protein was used.

(ii) Efflux and Exchange in Intact Cells—Cells were incubated overnight at 4 °C with the appropriate concentration of radiolabeled galactoside and in the presence of deoxyribonuclease I (20 μg/ml). The next day, potassium azide and carbonylcyanide *m*-chlorophenylhydrazone were added to final concentrations of 30 mM and 50 μM, respectively, and the cells were incubated for another 2 h at room temperature essentially as described (King and Wilson, 1990). Aliquots of 1 μl of concentrated cell suspension (50–80 mg/ml) were diluted into 500 μl of KPM or CPC buffer containing no substrates (efflux) and unlabeled galactosides (exchange), respectively. The transport reaction was stopped by rapid filtration as described above.

(iii) Efflux and Exchange in Membrane Vesicles—For efflux and exchange in the presence of artificially imposed diffusion potentials, membrane vesicles were resuspended to a final concentration of 30–40 mg of protein/ml in the buffers specified below prior to loading with 5 mM [^{14}C]lactose or 2 mM [^{14}C]methyl-β-D-thiogalactopyranoside. To generate a ΔΨ, valinomycin was added to the membrane suspension to a final concentration of 2 nmol/mg of protein. The membranes were washed and resuspended in 120 mM potassium P_i or 120 mM sodium phosphate (NaP_i), pH 8.0, containing 2 mM $MgSO_4$. Potassium-loaded membranes were diluted into the same buffer (no gradient) or in NaP_i buffer (ΔΨ, interior negative). Sodium-loaded membranes were diluted into the same buffer (no gradient) or in potassium-containing buffers (ΔΨ, interior positive).

Measurement of Proton Transport

Sugar-induced proton uptake was measured essentially as described by Henderson and Macpherson (1986). Cells were washed and resuspended in an equal volume of 150 mM KCl plus 2 mM glycylglycine, pH 6.5. The cells were starved for 3 h at 37 °C (shaking cell suspension), washed two more times, and resuspended in the KCl-glycylglycine medium to a final protein concentration of 60–70 mg/ml. Proton transport was measured with a pH electrode in 150 mM KCl, 30 mM potassium thiocyanate plus 2 mM glycylglycine, pH 6.2, at a cell density of 3 mg/ml. The cell suspension was made anaerobic by flushing with argon. Proton uptake was induced by addition of 5 mM sugar.

Immunoblotting

The amount of wild-type and various mutant LacS proteins in the membrane was estimated by immunoblotting with an anti-carboxyl terminus antibody directed against the synthetic peptide NH_2-Cys-Glu-Lys-Val-Glu-Ala-Leu-Ser-Glu-Val-Ile-Thr-Phe-Lys-Lys-Gly-Glu (COOH-terminal acid)). The synthetic peptide (Multiple Peptide Systems, San Diego) was conjugated to keyhole limpet hemocyanin through the cysteine at the amino terminus of the peptide (Harlow and Lane, 1988). Immunization of rabbits and collecting blood samples were performed using standard procedures (Harlow and Lane, 1988). Inside-out or right-site-out membrane vesicles of *E. coli* DW2 (or HB101) carrying plasmid pSKE8 and expressing wild-type or mutant LacS protein were used to estimate the expression of the lactose transport protein. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% polyacrylamide) and transferred to polyvinylidene difluoride membranes by semidry electrophoretic blotting. Processing of the polyvinylidene difluoride membranes (antibody binding: serum dilutions of 10,000–40,000-fold), and immunoblot detection using a secondary alkaline phosphatase-labeled anti-rabbit IgG was performed as described (Harlow and Lane, 1988).

Site-directed Mutagenesis and DNA Sequencing

The mutagenic primers used are E379A, 5′-CACCGTGATG**CT**-TCGTTAACTTTGTCA; E379D, 5′-CACCGTGATG**AT**TCGTTAACTTT-GTCA; and E379Q, 5′-CACCGTGAT**C**AATCGTTAACTTTGTCA. The

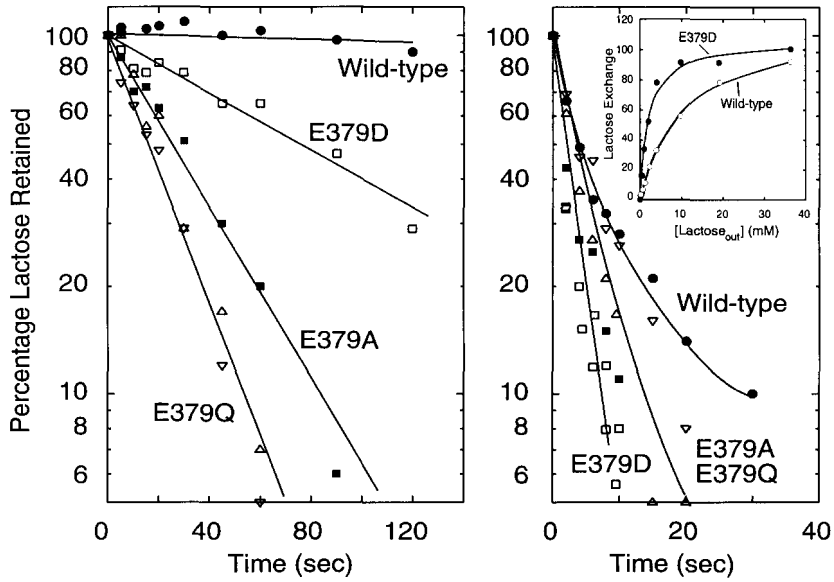

FIG. 5. **Efflux down a concentration gradient and equilibrium exchange of lactose by wild-type and mutant LacS proteins.** *E. coli* DW2/pSKE8 wild-type and various LacS mutants were grown in Luria broth supplemented with carbenicillin (50 μg/ml). Cells were washed and resuspended in KPM, pH 6.6, to a final protein concentration of 60–70 mg/ml. Loading of the cells with [^{14}C]lactose (5 mM, final concentration) was performed as described under "Experimental Procedures." Aliquots of cells (1 μl) were diluted 500-fold into KPM, pH 6.6, supplemented with 50 μM CCCP and without (downhill efflux) or with 5 mM lactose (equilibrium exchange). Efflux (*left panel*) and exchange (*right panel*) were assayed at 20 °C. *Inset*, non-equilibrium exchange of lactose by *E. coli* DW2/pSKE8 LacS (wild-type) and LacS-E379D. The assay temperature was kept at 10 °C. The experimental data were fitted to the Michaelis equation. Activity is given as nmol/min × mg of protein.

nucleotides changed by the site-directed mutagenesis are underlined. The nucleotide changes resulting in the desired amino acid substitution are indicated in bold. The other (silent) mutations were made to create a new *Hin*cII site. Site-directed mutagenesis was carried out by the Kunkel method (Kunkel *et al.*, 1987). Single-stranded uracil-containing DNA of pSKE8 was isolated from *E. coli* CJ236 (*dut*$^-$, *ung*$^-$)/pSKE8 after infection with helper phage M13KO7 (Sambrook *et al.*, 1989). Closed circular heteroduplex DNA with the desired mutations was synthesized *in vitro* as described (Kunkel *et al.*, 1987) and transformed to *E. coli* JM101 (*ung*$^+$). Plasmid DNA was isolated from a number of transformants and plasmids bearing the desired mutation(s) were identified by digestion with *Hin*cII. The plasmids with an extra *Hin*cII site were transferred to *E. coli* HB101, and the phenotype (growth on MacConkey-lactose agar and mineral media, transport activity) of at least two independent isolates was determined (see "Results"). Subsequently, mutations were verified by nucleotide sequencing of double-stranded DNA using the dideoxy chain termination method (Sanger *et al.*, 1977) and a set of primers complementary to a region of *lacS* located 50–100 bases downstream or upstream of the mutation site. The nucleotide sequence of the entire *lacS-E379Q* gene was determined.

RESULTS

Mutagenesis—Fig. 1 shows the alignment of the amino acid sequence of putative interhelix loop X-XI of the lactose transport protein (LacS) of *S. thermophilus* and homologous regions in other sugar transport proteins. The glutamate residue at position 379 of LacS is conserved in each of the aligned sequences (*arrow 3*). Glu^{379} of the lactose transport protein (LacS) was replaced by site-directed mutagenesis with aspartate, glutamine, or alanine. Each mutant was isolated independently at least twice, and the phenotypes were compared with those of the wild-type. Plasmids with the mutations were used to transform *E. coli* HB101 (*lacY1*), and transformants were streaked on lactose MacConkey agar plates or mineral medium plates supplemented with lactose. The *lac* phenotype of each of the position 379 mutants on both types of media was indistinguishable from that of the wild-type strain, *i.e.* HB101/pSKE8.

Uphill and Downhill Transport—To measure uphill transport of lactose, each of the plasmids, bearing *lacS* wild-type or a mutant allele, was used to transform *E. coli* DW2 (Δ*lacZY*). The wild-type protein accumulated lactose 13–14-fold within 4 min, LacS-E379D accumulated 2–3-fold, while no accumulation was observed with the mutants in which Glu^{379} was replaced by alanine or glutamine. The LacS-E379A and LacS-E379Q exhibited an initial rate of lactose uptake that was significantly higher than the strain with the plasmid control (Fig. 2, *left panel*).

For downhill uptake of lactose, *E. coli* HB101 was used as host, and cells were grown in the presence of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside in order to express the β-galactosidase activity maximally. Uptake of lactose down its concentration gradient was observed with LacS (wild-type) but also with the Glu^{379} mutants (Fig. 2, *right panel*). In fact, the rates of lactose uptake by the Glu^{379} mutants were significantly higher than those of the wild-type.

The relative amounts of LacS were estimated by immunoblotting with a anti-IIA domain antibody. Membranes prepared from strains expressing either the wild-type or one of the mutant LacS proteins showed no significant differences in the expression levels of LacS (data not shown).

Coupling of Lactose and H$^+$ Transport—The apparent inability of the LacS-E379Q and E379A mutants to transport lactose against a concentration gradient (Fig. 2, *left panel*) could be due to a defect in the coupling of lactose and proton transport. Lactose uptake by *E. coli* DW2/pSKE8 wild-type LacS and LacS-E379Q was measured for prolonged time intervals (up to 30 min, sampling every 1–2 min) and at pH 5.0, 5.7, 6.6, and 7.5. Fig. 3 shows that highest accumulation levels are observed at pH 5.7 ($lactose_{in}/lactose_{out}$ is 33 for wild-type LacS). For LacS-E379Q some accumulation can be observed at pH 5.0 and 5.7, but not at pH 6.6 and 7.5.

Lactose-induced H$^+$ uptake was measured with a pH electrode. Addition of 5 mM lactose to a suspension of *E. coli* DW2/pSKE8 wild-type LacS resulted in a transient alkalinization of the medium which is consistent with a lactose H$^+$ symport mechanism (Fig. 4). The alkalinization was reduced in the LacS-E379D mutant and was not observed with strains carrying either the control plasmid (LacS$^-$), LacS-E379A, or LacS-E379Q. From these experiments it is tentitatively concluded that lactose and H$^+$ uptake in the LacS-E379Q (and E379A) mutant(s) is uncoupled and that lactose uptake proceeds via uniport.

Efflux Down a Concentration Gradient and Equilibrium Exchange—The experiments presented above describe uphill (Δ*p*-driven) and downhill (driven by the lactose concentration gradient, ΔpLac; $[lactose]_{in} < [lactose]_{ex}$ due to high β-galactosidase activity) lactose uptake. The lactose transport protein also catalyzes reaction(s) in the opposite direction, *e.g.* efflux down a concentraton gradient as well as exchange (Foucaud and Poolman, 1992). Fig. 5 shows efflux and equilibrium exchange of lactose at pH 6.6 ($pH_{in} = pH_{out}$) by wild-type LacS

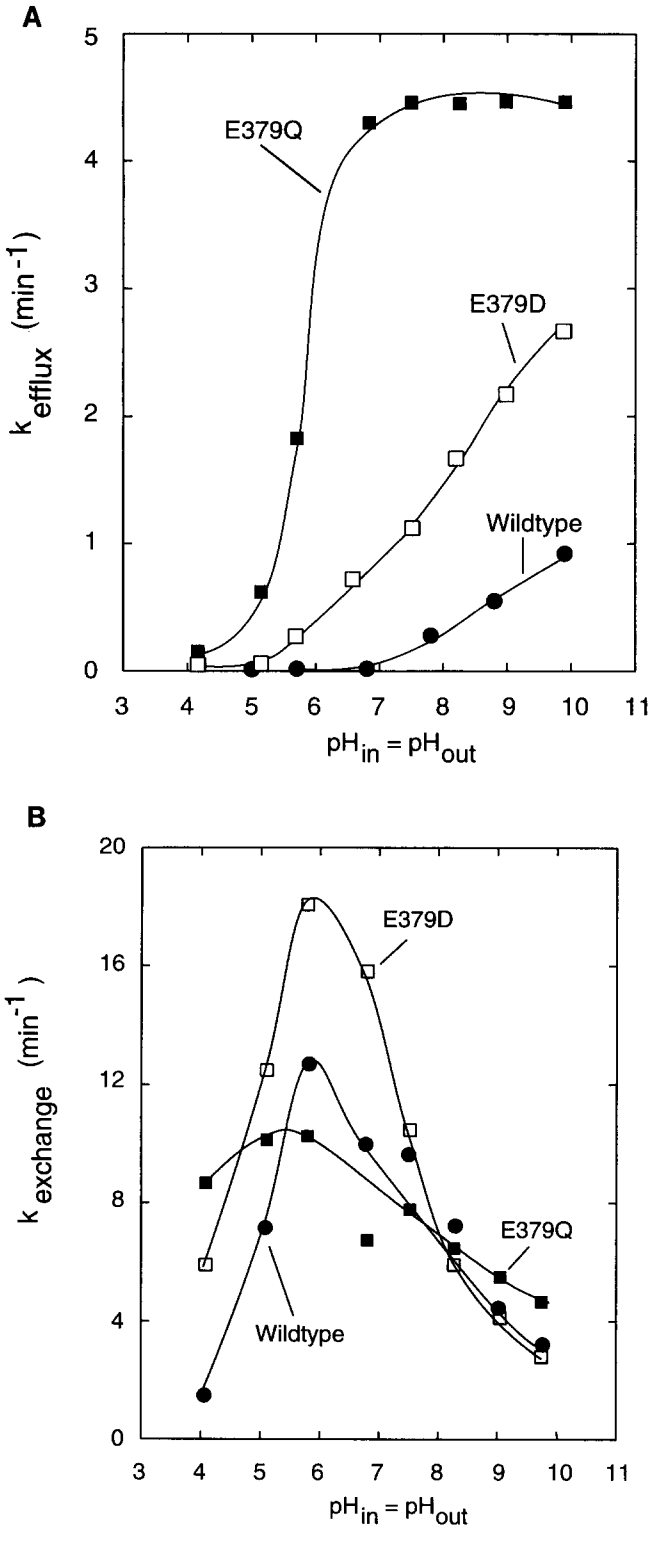

FIG. 6. **pH dependence of efflux down a concentration gradient (*A*) and equilibrium exchange (*B*) of lactose by wild-type and mutant LacS proteins.** Experimental procedures are the same as described in the legend of Fig. 5 except that CPC buffer was used and the external pH was varied between 4.1 and 9.8. The temperature was kept at 25 and 15 °C for the efflux and exchange assay, respectively. First-order rate constants of efflux (*A*) and exchange (*B*) are plotted as a function of pH.

and the various Glu^{379} mutants. Lactose efflux down a concentration gradient was slow for the wild-type protein, much faster for LacS-E379D, and fastest for LacS-E379A and LacS-E379Q (Fig. 5, *left panel*). This indicates that substitution of Glu^{379} for Asp, Ala, or Gln alters the rate-determining step(s) of the efflux reaction, possibly by shifting the protonation equilibrium at the outer surface of the membrane to the deprotonated form of the carrier molecule or allowing a more rapid return of the unloaded carrier to the inner surface of the membrane. The differences in the rates of lactose exchange catalyzed by the wild-type and mutant proteins were relatively small (Fig. 5, *right panel*), suggesting that the rate-determining steps in the translocation cycle for exchange are different from those in efflux transport.

Under conditions of exchange with 5 mM [^{14}C]lactose inside and 5 mM [^{12}C]lactose outside the cell, the exchange reaction of the Glu^{379} mutants was consistently faster than that of the wild-type. Since the release of [^{14}C]lactose from the cell is monoexponential, at least initially, with a slope that depends upon the external lactose concentration, an apparent K_m^{out} for the exchange reaction can be estimated. The K_m^{APP} values of the wild-type LacS and LacS-E379D were 10 and 2.5 mM, respectively (Fig. 5, *right panel, inset*). The maximal rates of exchange were the same for the wild-type and E379D mutant protein. Since the wild-type and Glu^{379} mutant proteins are expressed to the same level in *E. coli* membranes, the differences in the equilibrium exchange reactions shown in Fig. 5, *right panel*, can be explained by the lower K_m^{APP} of the E379D mutant relative to the wild-type protein.

pH Dependence of Efflux Down a Concentration Gradient and Equilibrium Exchange—The pH dependence of the first-order rate constant (k) of the efflux reaction of the wild-type, E379D, and E379Q mutant proteins is shown in Fig. 6*A*. The pH profile of the E379D mutant is shifted to lower pH values by at least 2 units, whereas the profile of the E379Q mutant is shifted even further (pK of about 6.0). This suggests that residues at position 379 directly affect the deprotonation of the carrier molecule at the outer surface of the membrane, perhaps by modulating the pK of a nearby group that is on the pathway of proton translocation.

Equilibrium exchange of galactosides in membrane vesicles of *S. thermophilus*, expressing LacS, exhibits an optimum between pH 6–7 (Foucaud and Poolman, 1992). Similar results were obtained with the product of the *lacS* gene cloned in *E. coli* (Fig. 6*B*). Moreover, the pH profile of the exchange reaction of the wild-type and E379D mutant LacS proteins was similar. In contrast to the wild-type protein and LacS-E379D, the E379Q mutant was much less affected by pH in the range of 4 to 10.

Effect of Membrane Potential on Efflux Down a Concentration Gradient and Equilibrium Exchange—Neutral substitutions of Glu^{379} result in a carrier protein that is unable to transport lactose against a concentration gradient. To test whether a membrane potential ($\Delta\Psi$) has a kinetic effect on the apparent uniport of lactose, efflux down a concentration gradient by the wild-type and E379Q mutant in the presence and absence of a $\Delta\Psi$ was compared. Fig. 7, *left panel*, shows, as expected, that lactose efflux in membrane vesicles bearing wild-type LacS is retarded by a $\Delta\Psi$ (inside negative). Comparable results were obtained with LacS-E379D (data not shown), but also (at least qualitatively) with membrane vesicles derived from strains expressing LacS-E379Q (Fig. 7, *right panel*). These results indicate that also for the LacS-E379D and LacS-E379Q enzymes one or more steps in the translocation cycle are affected (kinetically) by the membrane potential. Equilibrium exchange of lactose by LacS wild-type and position-379 mutants was not affected by the $\Delta\Psi$ (data not shown).

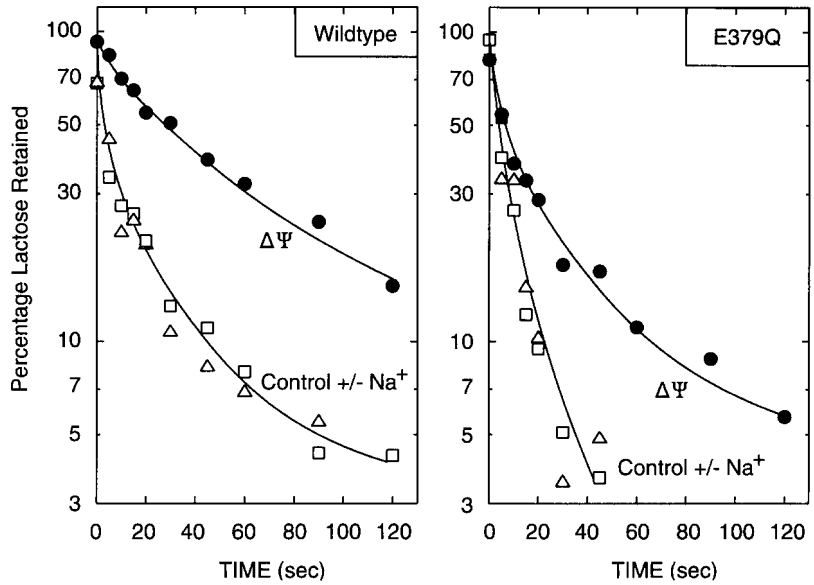

FIG. 7. **Effect of membrane potential (ΔΨ) and sodium gradient (ΔpNa) on lactose efflux down a concentration gradient in right-site-out membrane vesicles of *E. coli* DW2/pSKE8 expressing the wild-type or LacS-E379Q protein.** Membrane vesicles were washed and resuspended in 20 mM potassium phosphate, 100 mM potassium acetate, pH 8.0. Concentrated membrane vesicles (35 mg of protein/ml) were diluted 100-fold into the appropriate buffers, pH 8.0, to generate a ΔΨ (inside negative) or ΔpNa (control $+Na^+$, 20 mM NaCl on the outside) or no gradient (control $-Na^+$) as described under "Experimental Procedures." The internal lactose concentration was 5 mM, and efflux was assayed at 30 °C.

TABLE I
Properties of the LacS Glu^{379} and LacY Glu^{325} mutants

Parameter	Phenotype/activity/affinity[a]			
	LacS-E379D	LacS-E379A/Q	LacY-E325D	LacY-E325A/Q
MacConkey-Lac	Red	Red	Red	Red
Accumulation	10–20%	No	10–20%	No
Lac-H^+ symport[b]	Lower	No	ND[c]	No
Downhill uptake	Normal	Normal	Normal	Normal
Downhill Efflux	Faster, pK_A ↓	Faster, pK_A ↓ ↓	ND	No
Exchange[d]	Normal	pH independent	pK_A shift	Normal[e]
K_m^{APP} (exchange)	4× ↓	ND	ND	Normal
NPG-binding	ND	ND	ND	K_D^{APP} 2× ↑

[a] Properties of LacS Glu^{379} and LacY Glu^{325} mutants are relative to the corresponding wild-type proteins. For the LacY Glu^{325} mutants, the data were taken from Carrasco *et al.* (1980), Roepe and Kaback (1990), and Lolkema *et al.* (1991).
[b] Lactose-induced proton uptake as determined from pH recordings.
[c] ND, not determined.
[d] Equilibrium exchange of LacS wild-type exhibits a pH optimum at 6–7; equilibrium exchange of LacY wild-type is independent of pH in the range of 5–10 but becomes dependent on pH in the E325D mutant (decreases above pH 8) (Roepe and Kaback, 1990).
[e] pH dependence of exchange has not been analyzed in detail.

DISCUSSION

In this paper the effects on the kinetic parameters of transport of different substitutions of Glu^{379} in the lactose transport protein of *S. thermophilus* have been analyzed. The properties of LacS Glu^{379} mutants reveal some striking similarities and differences with the corresponding mutations in LacY (Table I). The mutations reduce the ability of both enzymes to accumulate sugars against a concentration gradient. Neutral substitutions abolish uphill transport completely whereas the Glu to Asp substitutions result in reduced accumulation levels. For both types of mutants, and both in LacS and LacY, the rate of downhill uptake is not much affected. Also, the apparent affinity constants for transport or sugar binding are only moderately affected by the Glu^{379} and Glu^{325} mutations in LacS and LacY, respectively.

The mutant alleles of both permeases differ in those aspects where also the properties of the wild-type enzymes are different. Equilibrium exchange of lactose mediated by the wild-type LacY protein is independent of pH in the range of 5–10 but becomes dependent on pH in the E325D mutant (Roepe and Kaback, 1990). Neutral substitutions result in wild-type exchange activities (Carrasco *et al.*, 1989). Equilibrium exchange of lactose by wild-type LacS and LacS-E379D exhibits a typical pH optimum around pH 6, whereas exchange by LacS-E379Q is largely independent of pH (Fig. 6*B*). The rate of efflux of lactose down a concentration gradient at alkaline pH values approaches the rate of exchange in wild-type LacY (Viitanen *et al.*, 1983), whereas in wild-type LacS the rate of efflux is much slower than the rate of exchange (Fig. 6). Remarkably, the rate of efflux is accelerated in the LacS-E379D, E379A, and E379Q mutants (Figs. 5 and 6*A*), whereas lactose efflux by the LacY-E325A and E379Q mutants is inhibited and only marginally greater than in membranes lacking LacY (Carrasco *et al.*, 1989). The apparent unidirectional transport by the LacY Glu^{325} mutants has not been fully explained but suggests that translocation of the unloaded carrier is highly rate-determining during downhill efflux but not during downhill influx.

Taken together, these results indicate that Glu^{379} in LacS and Glu^{325} in LacY play direct roles in the energy coupling mechanisms of the corresponding proteins and that the glutamate residues are closely associated with the pathway for proton transport in each of the proteins. Without the negative charge at position 379 (LacS) and 325 (LacY), the proteins behave as sugar uniporters at least when uptake transport is assayed. A similar role for Glu^{379} in LacS and Glu325 in LacY is remarkable because the overall polarity of the sequences around these residues is quite different, which, in turn, suggests a different position of the residues in the secondary structure models of the two proteins. The sequence motif Lys^{319}-*X*-*X*-His^{322}-*X*-*X*-Glu^{325} of LacY is present in the middle of putative transmembrane α-helix X, which is supported by a large number of *lacY-phoA* (LacY-alkaline phosphatase) fu-

sions (Calamia and Manoil, 1990, 1992). Given the similarity in function this would imply that the motif Lys^{373}-*X*-*X*-His^{376}-*X*-*X*-Glu^{379} of LacS is present in a similar transmembrane segment. However, the region around Lys^{373}, His^{376}, and His^{379} in LacS is much more hydrophilic (Fig. 1), and, hydropathy profiling predicts this region to be in a cytoplasmic loop between α-helices X and XII. The functional similarities between Glu^{379} of LacS and Glu^{325} of LacY (this study), and His^{376} of LacS and His^{322} of LacY (Poolman *et al.*, 1992), suggest that the hydrophilic region containing the motif Lys^{373}-*X*-*X*-His^{376}-*X*-*X*-Glu^{379} of LacS is located in the interior of the protein as well and forms part of the translocation pathway.

To specify which step(s) in the translocation cycle of the LacS protein are affected by the Glu^{379} mutations, the kinetic behavior of the sugar H^+ symporter has been simulated. The procedure for analyzing the steady state kinetic behavior of hypothetical enzymes has been described previously (Lolkema, 1993). The kinetics of the efflux and exchange reactions catalyzed by wild-type LacS have been studied extensively (Foucaud and Poolman, 1992; this work). For instance, exchange is characterized by a bell-shaped pH profile that has its optimum around pH 6. The pH optimum for efflux is at much higher pH values (>8) and, moreover, efflux is much slower than exchange. Based on these and other observations, it has been concluded that the binding order on the inside of the membrane is substrate first, proton second. Simulations of kinetic schemes with random and various orders of binding showed that the suggested ordered binding on the inside exclusively resulted in the observed experimental behavior, provided that binding at the outside was *not* of the same order (*i.e.* either random or proton first, substrate second) and that the pK of the proton binding site on the outside was at least 4 pH units higher than on the inside (not shown). The random order on the outside was chosen (see below) in the kinetic scheme presented in Fig. 8, *top panel*, that together with the set of rate constants described in the legend to the figure mimics wild-type LacS. The pH profile of exchange is a consequence of the ordered binding at the inner side of the membrane and the optimum is mainly determined by the pK of the proton binding site at this side of the membrane (pK_i = 6; Fig. 8, *top panel*). At pH values below the pK_i, the rate goes down because the carrier remains protonated at the inside which inhibits the exchange between bound and free substrate. At pH values above the pK_i, the exchange rate goes down because the carrier deprotonates which reduces the fraction of the enzyme in the ternary complex. The maximal rate of exchange at the pH optimum is determined by the rate constants for the translocation of the ternary complex (k_3 and k_{-3}). Efflux requires protonation of the carrier at the inside of the membrane (pK_i = 6) and deprotonation of the carrier at the outside (pK_o = 10). The two pK values involved are such that these requirements are difficult to meet, which results in low turnover rates. Maximal turnover is observed at a pH value in between the two pK values. At pH values below the optimum, the rate decreases because the carrier remains protonated at the external face of the membrane, whereas at pH values above the optimum, the rate decreases because the carrier does not become protonated at the internal face of the membrane.

In a previous report (Lolkema and Poolman, 1995), we analyzed the phenotype of uncoupled transport mutants in which either the binary enzyme-sugar complex or the enzyme-H^+ complex can freely reorient its binding sites (the "*E*S leak" and "*E*H leak," respectively). Mutants of Glu^{379} in LacS are of the *E*S leak type by the following criteria: (i) sugar accumulation increases with decreasing pH (Fig. 3), (ii) the rate of efflux increases sigmoidally with pH (Fig. 6*A*), and (iii) efflux is inhibited by the membrane potential (Fig. 7) under conditions where uphill transport cannot be observed (Figs. 2 and 3). Apparently, the Glu^{379} mutations induce the isomerization of the binary enzyme-sugar complex (Fig. 8, *bottom panel*).

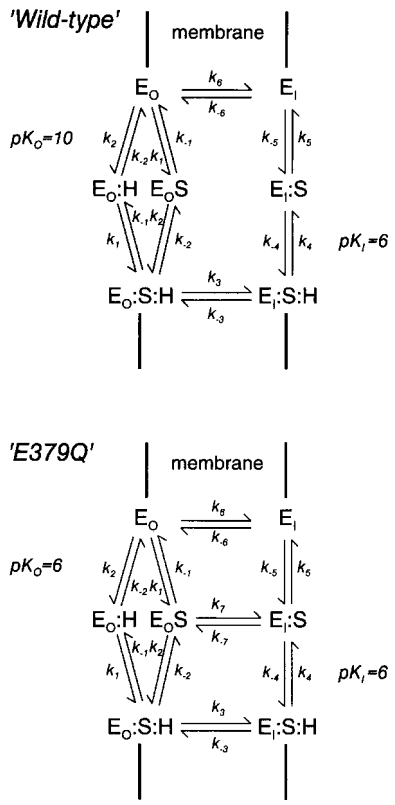

FIG. 8. **Schematic representation of the reactions involved in LacS-mediated galactoside transport.** *E*, H^+, and *S* represent the carrier protein, proton, and ligand (galactoside), respectively. The *subscripts o* and *i* refer to the outer and inner surface of the membrane, respectively. In the model, the formation of the ternary complex (ESH_o) via binary EH_o and ES_o are indicated; the association-dissociation of substrate and proton at the inner surface of the membrane is ordered (see text). Since information on cooperativity between H^+ and sugar binding is not available, the rate constants for H^+ binding to the free and sugar liganded carrier were chosen to be identical (likewise for sugar binding). A membrane potential was included in the simulations by affecting k_6, k_{-6}, k_7, and k_{-7} as described previously (Lolkema and Poolman, 1995). *Top panel*, wild-type LacS protein. The affinity constant for the substrate is 1 mM, the pK_{out} and pK_{in} are 10 and 6, respectively; the corresponding rate constant are: k_1 = 1,000 mM^{-1} s^{-1}, k_{-1} = 1,000 s^{-1}, k_2 = 100,000 μM^{-1} s^{-1}, k_{-2} = 10 s^{-1}, k_3 = 20 s^{-1}, k_{-3} = 200 s^{-1}, k_4 = 1,000 μM^{-1} s^{-1}, k_{-4} = 1,000 s^{-1}, k_5 = 1,000 mM^{-1} s^{-1}, k_{-5} = 1,000 s^{-1}, k_6 = 2000 s^{-1}, k_{-6} = 2 s^{-1}. *Bottom panel*, LacS-E379Q protein. The pK_{out} has been lowered from 10 to 6 and an *E*S leak pathway (translocations k_7, k_{-7}) has been introduced relative to the scheme for wild-type LacS; the following adjustments to the rate constants have been made: k_2 = 1,000 μM^{-1} s^{-1}, k_{-2} = 1,000 s^{-1}, k_{-3} = 20 s^{-1}, k_6 = 20 s^{-1}, k_{-6} = 20 s^{-1}, k_7 = 20 s^{-1}, k_{-7} = 20 s^{-1}. In order to lower the pK_{OUT}, both the k_2 and k_{-2} were altered; the behavior of LacS-E379Q can also be simulated by a similar change in pK which is achieved through adjustment of 1 of the rate constants. For the other equilibria we have chosen to alter both the forward and reverse rate constants. The changes in k_6, k_{-6}, and k_{-3} were made in order to meet the requirements of thermokinetic balancing (Walz and Caplan, 1988).

The membrane potential inhibits efflux transport of the wild-type LacS by affecting the $E_O \rightleftharpoons E_I$ transition. This transition is common to the pathway of coupled and uncoupled (uniport) transport (Fig. 8). To ascertain equilibration of internal and external substrate by the uniport pathway, the *E*S leak transition has to be $\Delta\Psi$ dependent as well. As a result, $\Delta\Psi$ affects the kinetics of the uniport cycle, but not the equilibrium position. A $\Delta\Psi$ (inside negative) slows down efflux through its effect on the $E_O \rightleftharpoons E_I$ step but accelerates efflux by affecting the

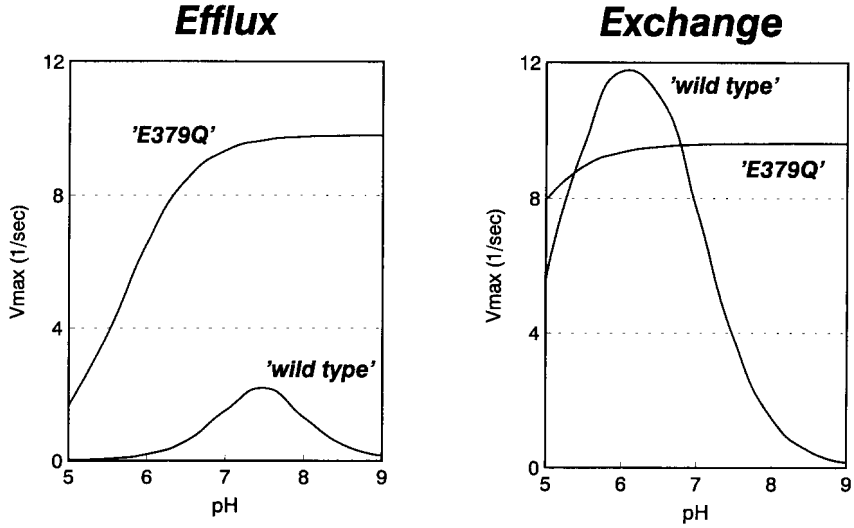

FIG. 9. **Simulation of the maximal rate of efflux down a concentration gradient and equilibrium exchange of LacS wild-type (Fig. 8, *top*)and LacS-E379Q (Fig. 8, *bottom*).** The maximal rates of efflux and exchange were analyzed as a function of pH using the kinetic parameters indicated in the legend to Fig. 8.

$ES_I \rightleftharpoons ES_O$ transition. The $E_O \rightleftharpoons E_I$ transition becomes more and more rate limiting as the $\Delta\Psi$ increases (Lolkema and Poolman, 1995), and, consequently, not only efflux by the wild-type but also that by an enzyme with an *E*S leak is inhibited by a $\Delta\Psi$ (inside negative).

The *E*S leak is most easily introduced in the scheme for the wild-type enzyme (Fig. 8, *top panel*) when the order of binding on the outside is assumed to be random instead of proton first, substrate second (see above). The pH profile of the efflux reaction catalyzed by the E379Q mutant (Fig. 6*A*) is indicative of a p*K* for the proton-binding site at the outer face of the membrane of around 6 (pK_o = 6). At pH values below the p*K*, the rate of efflux decreases because the carrier remains protonated at the outside. At higher pH values efflux proceeds via the uncoupled pathway involving translocation of the binary E:S complex which is independent of pH. Apparently, the E379Q mutation results in a lowering of the p*K* of the external proton-binding site. Simulation of the kinetics after introduction of the *E*S leak and lowering of the p*K* for proton binding on the outside results in the observed experimental phenomena, *i.e.* exchange that is largely independent of pH (Fig. 9, *right panel*), an efflux reaction that is stimulated relative to the wild-type enzyme (Fig. 9, *left panel*), uncoupled uptake above pH 6.5 and poorly coupled uptake below pH 6.5, and inhibition of efflux at high pH by a membrane potential, inside negative (not shown).

The analysis indicates that neutral substitutions of Glu^{379} in LacS lower the activation energy for translocation of the binary enzyme-sugar complex (*E*S leak) and lower the p*K* of the proton binding site at the outside from above 10 to 6, which is equal to the p*K* at the inside. The lowering of the pK_o suggests that in the wild-type enzyme the p*K* of the proton translocating residue(s) is modulated by Glu^{379}. Assuming a single site in the proton pathway, this would imply that Glu^{379} raises the p*K* of this site when the carrier is in the conformation with the binding sites facing outward. A lowering of p*K* in the mutants is consistent with the substitutions made ($Glu^{379} \rightarrow$ Gln and $Glu^{379} \rightarrow$ Ala), *i.e.* a negative charge raises the p*K* of a nearby group by stabilizing the protonated form. By removing the negative charge by mutation, or by a conformational change that is associated with the exposure of the binding sites to the inside, the p*K* is expected to be lowered. However, the observed shift in p*K* of more than 4 pH units upon substituting Glu^{379} for Gln is extremely large, and the shift may therefore not only be due to electrostatic effects (Russell and Fersht, 1987). In principle, a relatively minor conformational change in the E379Q mutant may have moved the essential protonatable group from a hydrophobic (low dielectric medium) to a more hydrophilic environment thereby lowering the p*K* even further. This would imply that in the wild-type enzyme the proton-binding site is in a more hydrophilic environment in the inside conformation than when it is on the outside. The properties of the E379D mutant are intermediate to those of the wild-type and E379Q protein which could reflect a different positioning of the carboxylate group relative to that in the wild-type protein. Finally, a single point mutation that results in a largely uncoupled transporter and a significant shift in p*K* suggests a mechanistic relation between coupling efficiency and the p*K* of the proton translocating group.

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